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4. Title of the invention

COMPOUND AND METHOD

5. Name of your agent *(if you have one)*

B.A.MARSH

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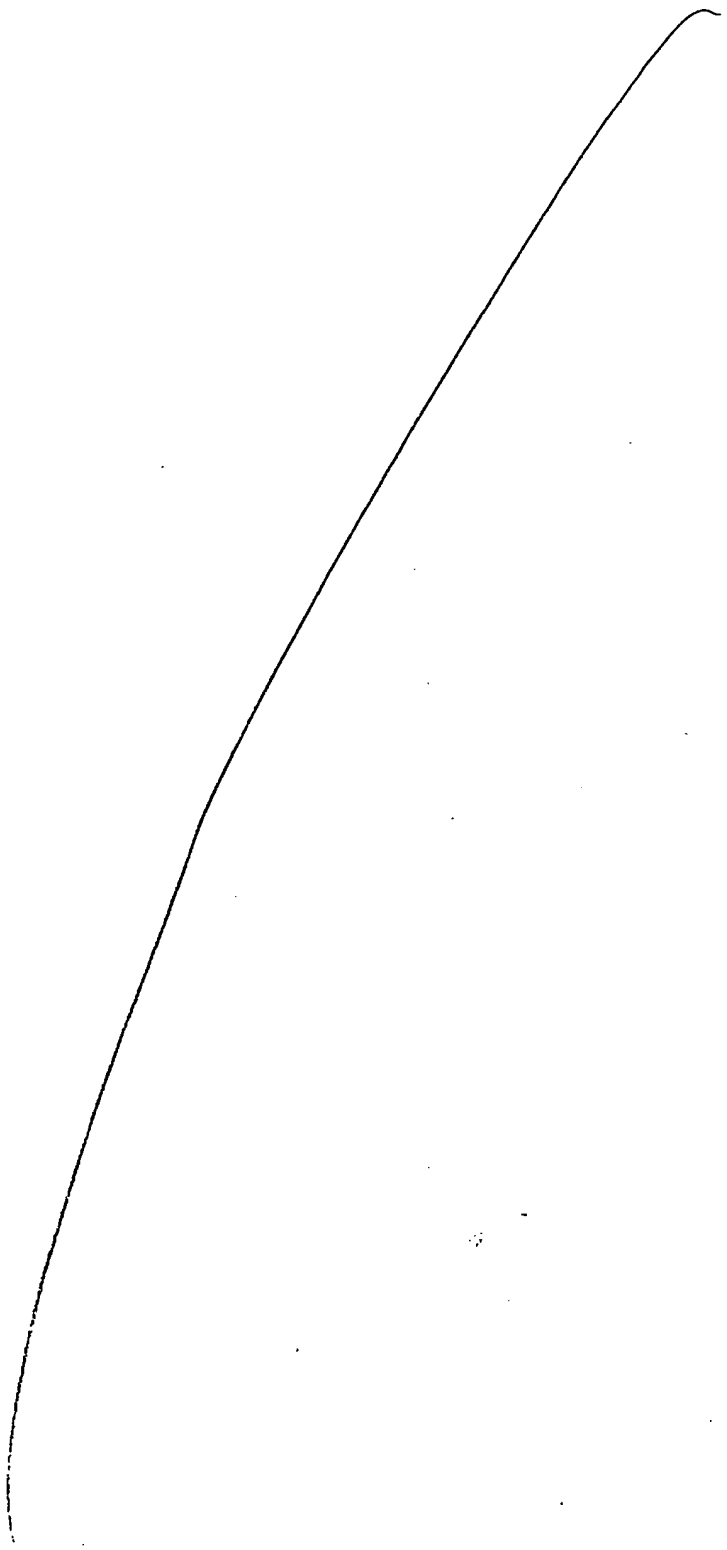

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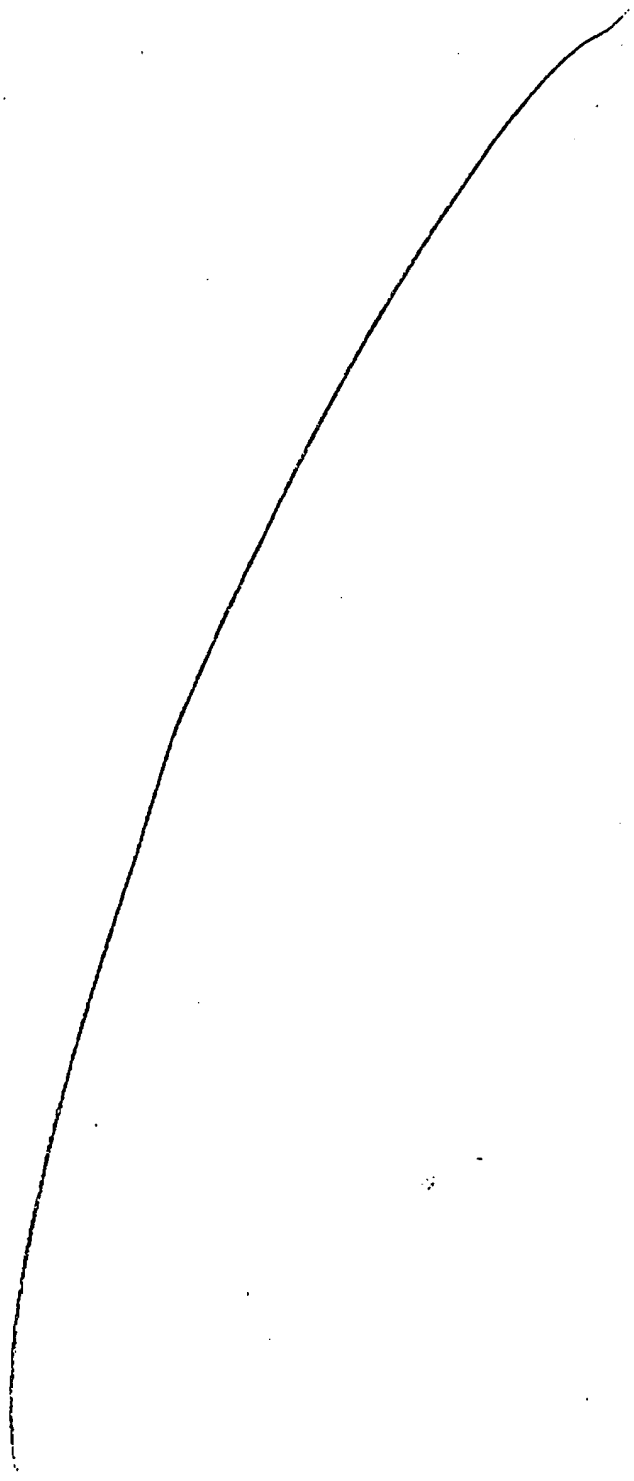

Compound and Method

The present invention relates to a compound which plays a pivotal role in transmission of intracellular signals from growth factor receptors. In particular, the invention describes a compound having an activity analogous to that of insulin in the regulation of glycogen synthesis and blood sugar levels, uses thereof in medicine, and to methods for screening for potential growth factor analogues and inhibitors.

Insulin is known to promote glycogen synthesis and concomitant lowering of blood sugar levels by inhibiting the activity of glycogen synthase kinase-3 (GSK3), which in active form inhibits glycogen synthase by phosphorylating it. We have now determined that the inactivation of GSK3 is directly carried out by the serine/threonine kinase Akt/RAC.

Akt/RAC is therefore implicated as the mediator of the physiological effects of growth factors in a number of distinct pathways. For example, Akt/RAC is implicated in the $p70^{S6K}$ pathway, acting at a level upstream of rapamycin/FKBP, in the PDE pathway and in numerous other signalling pathways involving growth factor receptors, especially those acting through PI3-K. Akt/RAC plays a pivotal role, connecting each of these pathways to external stimuli.

Accordingly, the invention provides an Akt/RAC peptide for use as a medicament. Moreover, the invention provides the use of an Akt/RAC peptide in the preparation of a pharmaceutical composition for the treatment of diseases associated with insulin deficiency or excess blood sugar levels.

The invention further provides the use of Akt/RAC as a tool for research into signalling pathways, for example as described hereinbelow.

The activity of Akt/RAC can be regulated, for example using agents such as vanadate. Vanadate is known for medical use, but its effect on Akt/RAC is unknown. Accordingly, the invention provides for the use of Akt/RAC regulating agents, for example vanadate, in the manufacture of compositions for use in medicine. The conditions which can be targeted

with such compositions include diseases involving excessive cell proliferation, such as tumours. Moreover, the compositions will be useful as immunosuppressive agents.

The invention provides a method for screening candidate growth factor analogues and inhibitors as defined above comprising the steps of:

(a) incubating an Akt/RAC peptide or a functional fragment or equivalent thereof with the candidate insulin analogue; and

(b) assessing the activity of the Akt/RAC peptide.

In the case of insulin, activators of Akt/RAC are candidate insulin analogues as they have the potential to lead to the inactivation of GSK3.

Activity of Akt/RAC may be assessed for example by incubating it with a substrate therefor and assessing the level of phosphorylation of the substrate. Alternatively, the phosphorylation level of Akt/RAC is measured directly. Preferably, the phosphorylation of residues known to be involved in Akt/RAC activation is measured. Such residues are, for example, threonine residues.

The invention is exemplified in the following manuscript document.

Glycogen synthase kinase-3[1] (GSK3) is implicated in the regulation of several processes important for mammalian cell physiology, including control of both glycogen metabolism[2] and protein synthesis[3] by insulin and modulation of the activity of transcription factors, such as AP-1 and CREB[4-6]. A *Drosophila* GSK3 homologue (*shaggy*) plays a crucial role in the *wingless* signaling pathway for the specification of cell fate[7,8]. GSK3 is inhibited by serine phosphorylation in vivo after stimulation of cells with insulin[3,11,12]. GSK3 can also be inhibited in vitro by serine phosphorylation catalysed by either MAP kinase-activated protein (MAPKAP) kinase-1 (also known as $p90^{rsk}$) or by p70 ribosomal S6 kinase ($p70^{S6K}$)[9,10]; these enzymes lie in distinct signalling pathways which are known to be stimulated by insulin and growth factors. We show here, however, that agents which prevent the activation of MAPKAP kinase-1 and $p70^{S6K}$ do not block insulin-induced phosphorylation and inhibition of GSK3 in the rat skeletal muscle cell line L6. We were able to identify another insulin-stimulated protein kinase in these cells that inactivate GSK3 under these conditions, which we demonstrate is the proto-oncogene Akt/RAC. Like the inhibition of GSK3[11-13], the activation of Akt/RAC is prevented by inhibitors of phosphatidylinositol (PtdIns) 3-kinase.

The activation of p42 MAP kinase and MAPKAP kinase-1 by insulin were suppressed completely by prior incubation of L6 myotubes with either 8-bromo-cyclic AMP (Fig 1A), or by PD 98059 (Fig 1C) a specific inhibitor of the activation of MAP kinase kinase[14]. In contrast, the 40-50% inhibition of GSK3 induced by insulin was unaffected by these agents (Figs 1B, 1D). Rapamycin, a specific inhibitor of the activation of $p70^{S6K}$ (Ref 15), also had no effect on the

inhibition of GSK3 by insulin in the absence[12,13] or presence (Fig 1D) of PD 98059. The failure of PD 98059, 8-bromo cyclic AMP and rapamycin to prevent the inactivation of GSK3 by insulin demonstrates that neither MAPKAP kinase-1 nor $p70^{S6K}$ is essential for this process. However, coexpression with MAPKAP kinase-1α in HeLa S3 cells enhances the phosphorylation and inhibition of GSK3β, particularly after phorbol ester stimulation[16], and in A431 cells the inhibition of GSK3α and GSK3β by EGF resulting from the phosphorylation of these isoforms at Ser-21 and Ser-9, respectively[17], is largely suppressed by expression of a dominant negative mutant of MAP kinase kinase-1[18]. MAPKAP kinase-1 may therefore play a role in the inhibition of GSK3 by growth factors or phorbol esters, which are much more potent activators of the MAP kinase pathway than is insulin.

In order to identify the kinase which inhibits GSK3 in the presence of rapamycin and PD 98059, L6 myotubes were incubated with both compounds and then stimulated with insulin. The cell lysates were chromatographed on Mono Q and the fractions assayed with the synthetic peptide GRPRTSSFAEG, termed here "Crosstide". This peptide corresponds to the sequence surrounding the serine (in bold type) whose phosphorylation triggers the inactivation of GSK3α(Ser-21)[10] and GSK3β(Ser-9)[9]. Three peaks of Crosstide kinase activity were eluted at 0.24 M, 0.28 M and 0.31 M NaCl (Fig 2A). These peaks were absent if insulin was omitted, or if the cells were incubated with 0.1 μM wortmannin prior to the addition of insulin (Fig 2A). The inhibition of GSK3 by insulin is also prevented by this concentration of wortmannin[12,13], or by 100 μM LY 294002 (Fig 1D), a structurally unrelated inhibitor of PI 3-kinase[19].

Akt-1/RACα and Akt-2/RACβ are protein serine/threonine kinases which are the cellular homologues of the viral oncogene v-akt. They are expressed ubiquitously in mammalian cells[20-25] and Akt-2/RACβ is overexpressed and amplified in 10% of ovarian neoplasms[25]. The catalytic domains are related most closely to the second messenger-dependent enzymes, cyclic AMP-dependent protein kinase and protein kinase C (almost 70% similarity). Recently, Akt/RAC was found to be activated by stimulation of NIH 3T3, Rat-1 or Swiss 3T3 cells with insulin or other growth factors, activation being prevented by wortmannin and unaffected by rapamycin[26-28]. All three peaks of Crosstide kinase from Mono Q (Fig 2A) could be immunoprecipitated by an anti-

Akt/RAC antibody (data not shown) and contained immunoreactive species which migrated on SDS-PAGE with apparent molecular masses of 58-60 kDa, slightly larger than E. coli-expressed human Akt-1/RACα (Fig 2B). The first and second peaks contained predominantly the 60 kDa species and the third mainly the 58 kDa species and a 59 kDa component. In contrast, an immunoprecipitating anti-MAPKAP kinase-1 antibody[12] failed to deplete any Crosstide kinase activity from the Mono Q fractions. Thus the three peaks are likely to represent isoforms of Akt/RAC in differing states of phosphorylation.

Akt/RAC was inactive in lysates prepared from unstimulated L6 myotubes, but was activated by insulin with a $t_{0.5}$ of <1 min. No activation of Akt/RAC occured if the myotubes were incubated with wortmannin or with LY 294002 prior to stimulation with insulin (Fig 2C). In contrast, there was almost no activation of MAPKAP kinase-1 (Fig 2D) by insulin after 2 min, the $t_{0.5}$ for being about 5 min (Fig 2D). The activation of $p70^{S6K}$ by insulin was even slower ($t_{0.5}$=5-10 min, data not shown). The faster activation of Akt/RAC as compared to p42 MAP kinase is similar to observations made previously in NIH 3T3 or Rat-1 cells after stimulation with PDGF[27,28] and is in line with the rate of inactivation of GSK3. In L6 myotubes[12] or Swiss 3T3 cells (Y.Saito and P.C., unpublished), the inhibition of GSK3 reaches a plateau value of 40-50% inhibition after 5 min, but inhibition is already 50-70% complete after 2 min. The activation of Akt/RAC by insulin was not prevented by 8-bromo-cyclic AMP or PD 98059 plus rapamycin (Fig 2C), consistent with the lack of effect of these agents on the inhibition of GSK3 by insulin (Figs 1B and 1D).

Akt/RAC immunoprecipitates from insulin-stimulated myotubes, potently inhibited GSK3α and GSK3β and inhibition was reversed by incubation with PP2A (Fig 3), demonstrating that it was triggered by serine/threonine phosphorylation of GSK3. In contrast, Akt/RAC immunoprecipitated from unstimulated cells, or cells incubated with wortmannin prior to stimulation with insulin, did not inactivate GSK3 (Fig 3). No inactivation of GSK3 occurred if the anti-Akt/RAC antibody was incubated with peptide immunogen prior to immunoprecipitation. Akt/RAC immunoprecipitates phosphorylated Crosstide at the serine, equivalent to Ser-21 in GSK3α and Ser-9 in GSK3β (data not shown).

Several lines of evidence indicate that PtdIns 3-kinase lies "upstream" of Akt/RAC. Firstly, the

activation of Akt/RAC is blocked by wortmannin[26-28] and LY 294002 (Fig 2C, Fig 3). Secondly Tyr-740 and Tyr-751 of the PDGF receptor, whose phosphorylation is critical to generate a high affinity binding site for the p85 subunit of PtdIns 3-kinase[26,27], are both required to activate Akt/RAC. Thirdly, PDGF-induced activation of Akt/RAC is prevented by a deletion mutant of the p85 subunit which inhibits receptor-induced PtdIns 3-kinase activity[27]. These observations suggest that the activation of Akt/RAC is likely to be triggered, directly or indirectly, by PtdIns (3,4,5)P_3, the putative second messenger generated by PI 3-kinase. It is therefore intriguing that the N-terminal pleckstrin homology domain of Akt/RAC binds PtdIns (3,4,5)P_3 at submicromolar concentrations, but since PtdIns(4,5)P_3 binds with similar affinity (M.Frech and B.A.H., unpublished experiments), the relevance of these observations is not yet clear. It has been reported that Akt/RAC is activated directly by PtdIns (3)P in vitro, but not by PtdIns or PtdIns(4,5)P_3[26], but an effect of PtdIns (3,4,5)P_3 on activity has yet to be demonstrated. However, activated Akt/RAC is phosphorylated on serine residues[27] and inactivated by intestinal alkaline phosphatase[27] or PP2A[28] (D.A.E.C., D.R.A. and P.C.unpublished), indicating that serine/threonine phosphorylation is also essential for activity. The activation of Akt/RAC might therefore be catalysed by a distinct PtdIns (3,4,5)P_3-dependent protein kinase. Alternatively, the interaction of Akt/RAC with PtdIns (3,4,5)P_3 might cause its recruitment to the plasma membrane where it is activated by another membrane-associated kinase. PtdIns (3,4,5)P_3 could also stimulate the autophosphorylation of Akt/RAC, converting it to an active, PtdIns (3,4,5)P_3-independent form. The activation of Akt/RAC by PDGF in NIH 3T3 cells was reported to be prevented by expression of dominant negative (Asn-17) Ras[26], but others failed to observe any effect of Ras^{asn17} or dominant-negative Raf on Akt/RAC activation by PDGF in Rat-1 cells, or by insulin or EGF in NIH-3T3 cells[27].

GSK3 phosphorylates serine residues in glycogen synthase whose dephosphorylation underlies the stimulation of glycogen synthesis by insulin in skeletal muscle[2]. The 40-50% inhibition of GSK3 by insulin, coupled with a similar activation of the phosphatase which dephosphorylates glycogen synthase[29] is therefore adequate to account for the two to three-fold stimulation of glycogen synthase by insulin in skeletal muscle[2] or L6 cells[30]. The stimulation of glycogen synthase and glycogen synthesis by insulin in L6 cells, like the inhibition of GSK3, is sensitive to

wortmannin and insensitive to PD 98059[30].

GSK3 is the first physiological substrate for Akt/RAC to be identified, but whether Akt/RAC is important in regulating other processes in which GSK3 has been implicated, such as cell fate determination[7,8] and protein synthesis[3] remains to be evaluated. Nevertheless, additional roles for Akt/RAC are suggested by the observation that its overexpression in Swiss 3T3 or COS 1 cells[28] or expression of a constitutively active Gag-Akt/RAC fusion protein in Rat 1 cells[27], triggers the activation of $p70^{S6K}$. However, $p70^{S6K}$ is activated rather slowly by insulin in L6 cells ($t_{0.5}$ = 5-10 min), compared to the activation of Akt/RAC ($t_{0.5}$ = 1 min) or the inactivation of GSK3 ($t_{0.5}$=2 min)[12]. Hence the effect of Akt/RAC on $p70^{S6K}$ may not be direct.

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Figure legends.

Fig.1. The inhibition of GSK3 by insulin in L6 myotubes is unaffected by agents which prevent activation of the classical MAP kinase pathway. The results are given as ± SEM for at least three different experiments. (A) The activation of p42 MAP kinase or MAPKAP kinase-1β (the major MAPKAP kinase-1 isoform in L6 cells) by insulin is prevented by 8-bromo cyclic AMP (8Br-cAMP). L6 myotubes were incubated with 8Br-cAMP (15 min) and then with insulin (5 min). Each enzyme was assayed after immunoprecipitation from cell lysates with specific antibodies, and the results are presented relative to the activities obtained in the presence of insulin and absence of 8Br-cAMP which were 0.04 ± 0.005 U/mg (p42 MAP kinase, SEM n=6) and0.071± 0.004 U/mg (MAPKAP kinase-1β, SEM n=6). (B) The inhibition of GSK3 by insulin is unaffected by 8Br-cAMP. The experiment was carried out as in A. Both GSK3 isoforms (GSK3α, GSK3β) were coimmunoprecipitated from the cell lysates and assayed before (filled bars) and after (open bars) reactivation with PP2A. The results are given relative to GSK3 activity in

lysates from unstimulated cells which was 0.08 ± 0.006 U/mg (SEM, n = 10). (C) The activation of p42 MAP kinase and MAPKAP kinase-1β is prevented by PD 98059 or LY 294002. L6 myotubes were incubated in the presence or absence of PD 98059 (1 h) or LY 294002 (10 min,, stimulated with insulin (5 min) and enzyme activities measured as in A. The activities of p42 MAP kinase and MAPKAP kinase-1β in the absence of insulin and presence of LY 294002, were identical to those shown in the absence of insulin and presence ofPD 98059 (D,E) The inhibition of GSK3 by insulin is unaffected by rapamycin and PD 98059, but prevented by LY 294002. In D, L6 myotubes were stimulated with insulin for the times indicated, and GSK3 activity was measured as in B, before (closed circles) and after (open circles) reactivation with PP2A. The closed triangle shows experiments in which the L6 myotubes were incubated with LY 294002 prior to stimulation with insulin and GSK3 assayed without PP2A treatment. In E, cells were incubated with rapamycin (triangles) or rapamycin plus PD 98059 (circles) prior to stimulation with insulin, and GSK3 activity measured as in B before (closed symbols) and after (open symbols) pretreatment with PP2A.

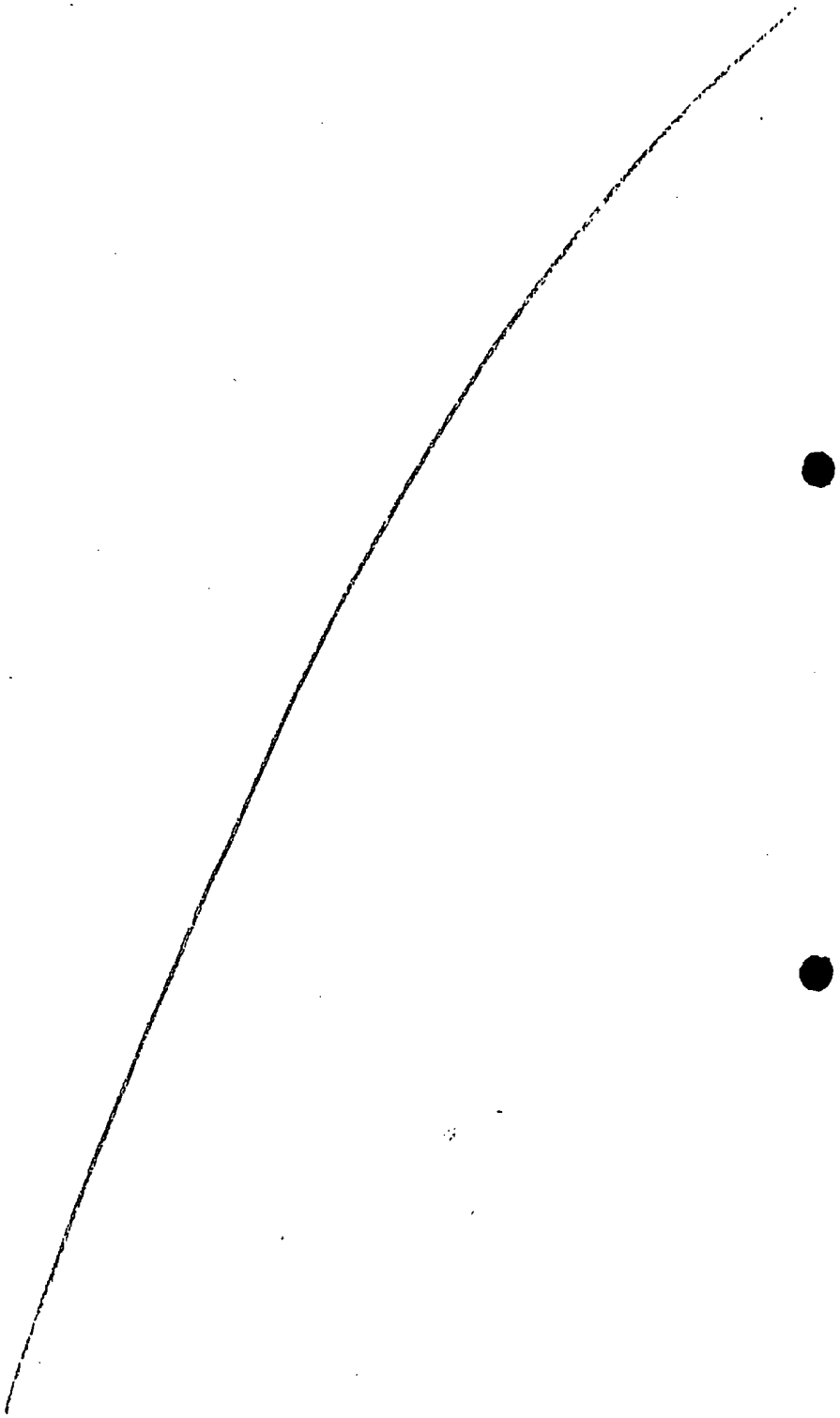
Methods. Monolayers of L6 cells were grown in 6 cm petri dishes to the stage of myotubes. deprived of serum overnight and then incubated for 1 h in 20 mM Hepes/NaOH (pH 7.4) / 0.14M NaCl / 5 mM KCl / 2.5 mM $MgSO_4$ / 1mM $CaCl_2$ / 25 mM glucose (HBS buffer)[12], in the presence or absence of 50 μM PD 98059 or 100 μM LY 294002. 2 mM 8Br-cAMP or 0.1 μM rapamycin, when added, were included for the last 15 min. The cells were stimulated for five min with 0.1 μM insulin (A, B, C) or for the times indicated (D, E), the medium removed by aspiration, placed on ice and lysed in 0.2 ml of ice-cold Buffer A [50 mM Tris-HCl (pH 7.5, 20°C) / 1 mM EDTA / 1 mM EGTA / 1% (w/v)Triton X-100 / 1 mM sodium orthovanadate / 10 mM sodium glycerophosphate / 50 mM sodium fluoride/ 5 mM sodium pyrophosphate / 2 μM microcystin, 0.1% (v/v) 2-mercaptoethanol/ leupeptin 4 μg/ml, 1 mM benzamidine, 1 mM phenylmethane sulphonyl fluoride, 30 μg/ml aprotinin, 30 μg/ml antipain, 10 μg/ml pepstatin. p42 MAP kinase, MAPKAP kinase 1β or (GSK3α plus GSK3β) were then immunoprecipitated from the cell lysates and assayed with specific protein or peptide substrates as in Ref 12. One Unit of protein kinase activity was that amount which catalysed the phosphorylation of 1 nmole of substrate in one min. Where indicated, GSK3 in immunoprecipitates was reactivated with PP2A as described[12].

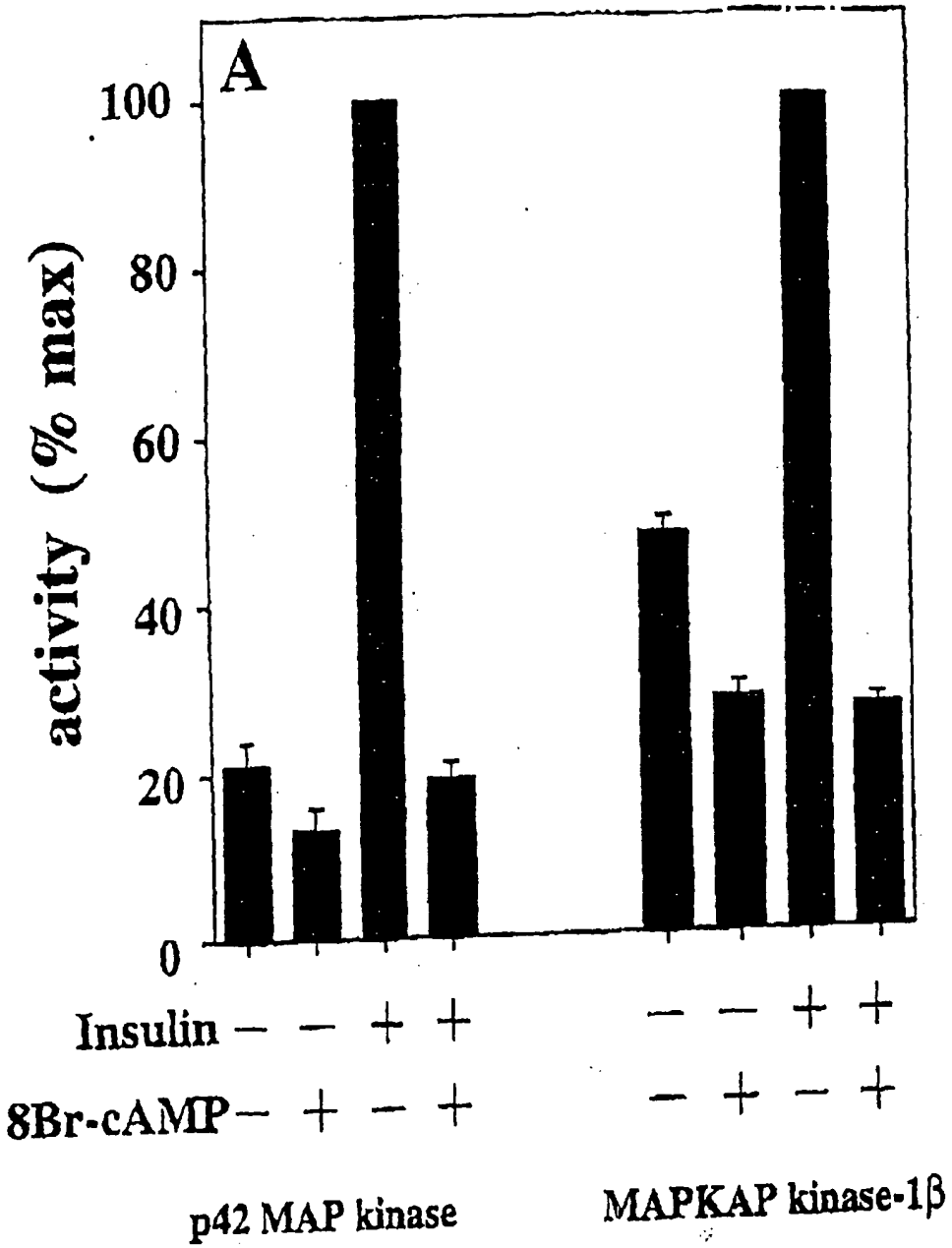
Fig 2. Identification of different forms of Akt/RAC as the insulin-stimulated, wortmannin-sensitive and PD 98059/rapamycin-insensitive Crosstide kinases in L6 myotubes. (A) L6 myotubes were incubated for 1 h in the presence of both 50 μM PD 98059 and 0.1 μM rapamycin, stimulated for 5 min with 0.1 μM insulin and lysed as in Fig 1. The lysates (0.3 mg protein) were chromatographed on a (5 x 0.16 cm) column of Mono Q. Fractions (0.05 ml) were collected and assayed for Crosstide kinase activity (closed circles). The open circles show a separate experiment in which insulin was omitted and the closed triangles an experiment in which wortmannin (0.1 μM) was added ten minutes before insulin. The NaCl gradient is shown by the broken line. Similar results were obtained in six different experiments. (B) Aliquots (10 μl) of the three peaks of Crosstide kinase activity from A (1, 2 and 3) and recombinant Akt/RAC (Akt) were electrophoresed on a 15% SDS/polyacrylamide gel and immunoblotted with an anti-Akt/RAC antibody. The positions of the marker proteins, myosin heavy chain (205 kDa), glycogen phosphorylase (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa) are indicated. (C) L6 myotubes were stimulated with 0.1 μM insulin for the times indicated and Akt/RAC was immunoprecipitated from the lysates (100 μg protein) and assayed for Crosstide kinase activity (open circles). The open triangles and open squares show, respectively, experiments in which the myotubes were preincubated with rapamycin plus PD 98059 or with 8Br-cAMP prior to stimulation with insulin. The closed circles and closed triangles show, respectively, experiments in which the myotubes were incubated with wortmannin or LY 294002 prior to stimulation with insulin. Similar results were obtained in three different experiments and all assays were carried out in triplicate. (D) The experiment was carried out as in C, except that MAPKAP kinase-1β was immunoprecipitated from the lysates and assayed with S6 peptide (closed circles). The closed triangles and open circles show, respectively, separate experiments in which the myotubes were incubated with rapamycin plus PD 98059 or with 8Br-cAMP prior to stimulation with insulin. Similar results were obtained in three different experiments and all assays were carried out in triplicate.

Methods. The Mono Q column in A was equilibrated and run as described[10], except that the buffer also contained 1.0 mM EGTA, 0.1 mM sodium orthovanadate and 0.5% (w/v) Triton X-100. Anti-Akt/RAC antibodies were raised in rabbits against the peptide FPQFSYSASSTA and affinity purified[17]. This sequence corresponds to the C-terminal 12 residues of Akt-1/RACα, the first eight residues also being found in Akt-2/RACβ (residues 470-477). Immunoblotting was carried out as described[31]. L6 lysates (100μg protein, about 60 μl) were incubated for 2 h with 5 μl of Protein G-Sepharose conjugated to 20 μl of affinity-purified anti-Akt/RAC antibody (24 μg/ml). The suspension was centrifuged for 1 min at 13,000 x g, the supernatant discarded and the immunoprecipitate washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, three times with 50 mM Tris-HCl (20°C) / 0.1 mM EGTA / 0.03% (w/v) Brij 35 / 0.1% (v/v) 2-mercaptoethanol, and then assayed for Crosstide kinase activity (using 30 μM Crosstide), exactly as described for the assay of MAPKAP kinase-1[9]. One Unit of Crosstide kinase was that amount which catalysed the phosphorylation of 1 nmole of Crosstide in one min. Akt/RAC immunoprecipitates were treated with protein phosphatases as in Ref 12. Other experimental details are given in Fig 1.

Fig 3. GSK3 is inactivated by Akt/RAC immunoprecipitated from insulin-stimulated L6 myotubes. L6 myotubes were stimulated for 5 min with 0.1 μM insulin as in Fig 1 and Akt/RAC immunoprecipitated from 100 μg of cell lysate as in Fig 2. The immunoprecipitate was then used to inactivate GSK3 isoforms as described for the inactivation of GSK3 by MAPKAP kinase-1[9,10]. The filled bars show GSK3 activity measured after incubation with MgATP and Akt/RAC as a % of the activity obtained in control experiments where Akt/RAC was omitted. In the absence of Akt/RAC, GSK3 activity was stable for the duration of the experiment. The open bars show the activity obtained after reactivation of GSK3 with $PP2A_1$. The figure shows that no inactivation of GSK3 occurs if insulin is omitted, or wortmannin (0.1 μM) is added 10 min prior to insulin, or if the anti-Akt/RAC antibody is incubated with peptide immunogen (0.5 mM) prior to immunoprecipitation. Inactivation of GSK3α stops at 70% inhibition because these preparations contain small amounts of a truncated 48 kDa species that cannot be inactivated by phosphorylation [10]. The results are shown as ± SEM for three experiments (each carried out in triplicate).

Methods. GSK3α and GSK3β were partially purified from rabbit skeletal muscle[9,10] and assayed with a specific peptide substrate[12]. Each GSK3 isoform was diluted to 15 U/ml and GSK3 activity measured after incubation for 20 min with MgATP in the presence or absence of Akt/RAC. The incubation was then made 20 mM in EDTA to stop the kinase reaction, incubated for 20 min with 5 mU/ml $PP2A_1$ to reactivate GSK3[9,10], made 2 μM in okadaic acid to inactivate $PP2A_1$ and then assayed for GSK3 activity. There was no reactivation in control experiments in which okadaic acid was added before $PP2A_1$. $PP2A_1$ was partially purified from rabbit skeletal muscle and one unit of activity was that amount which catalysed the dephosphorylation of 1 nmole of glycogen phosphorylase in one min[32]. Other experimental details are described in Figs 1 and 2.




A
100
80
60
40
20
0
activity (% max)
Insulin – – + + – – + +
8Br-cAMP – + – + – + – +
p42 MAP kinase
MAPKAP kinase-1β

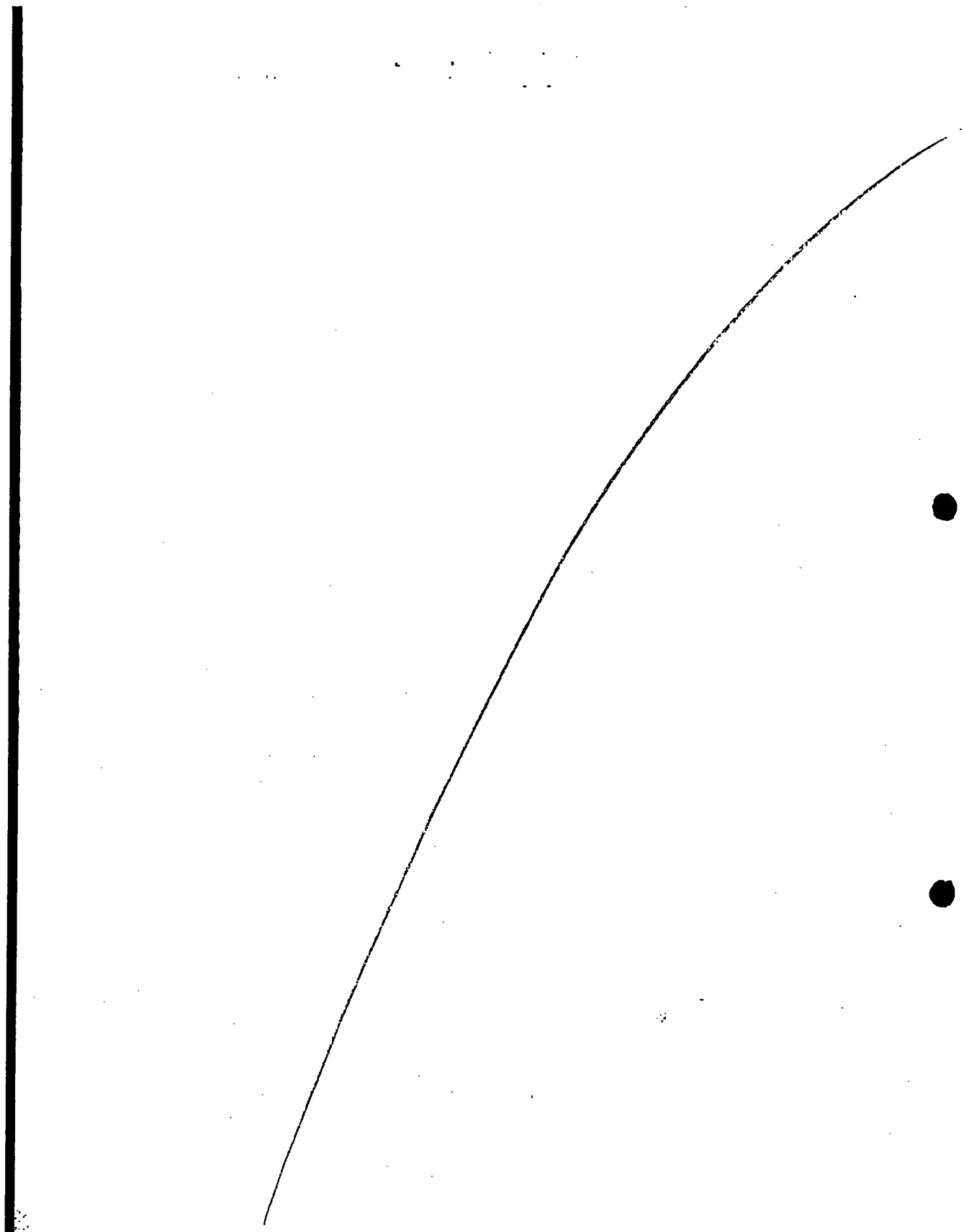
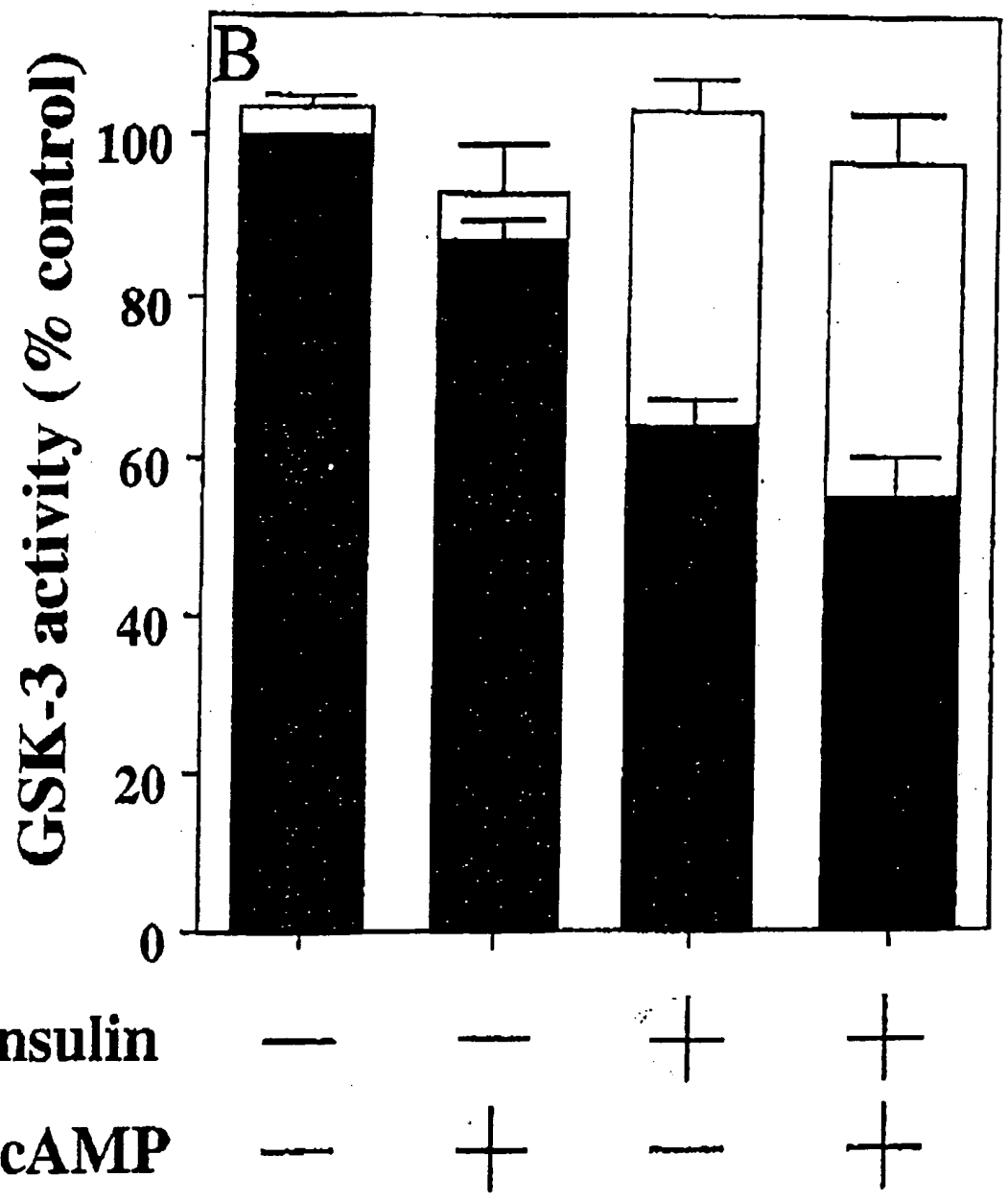
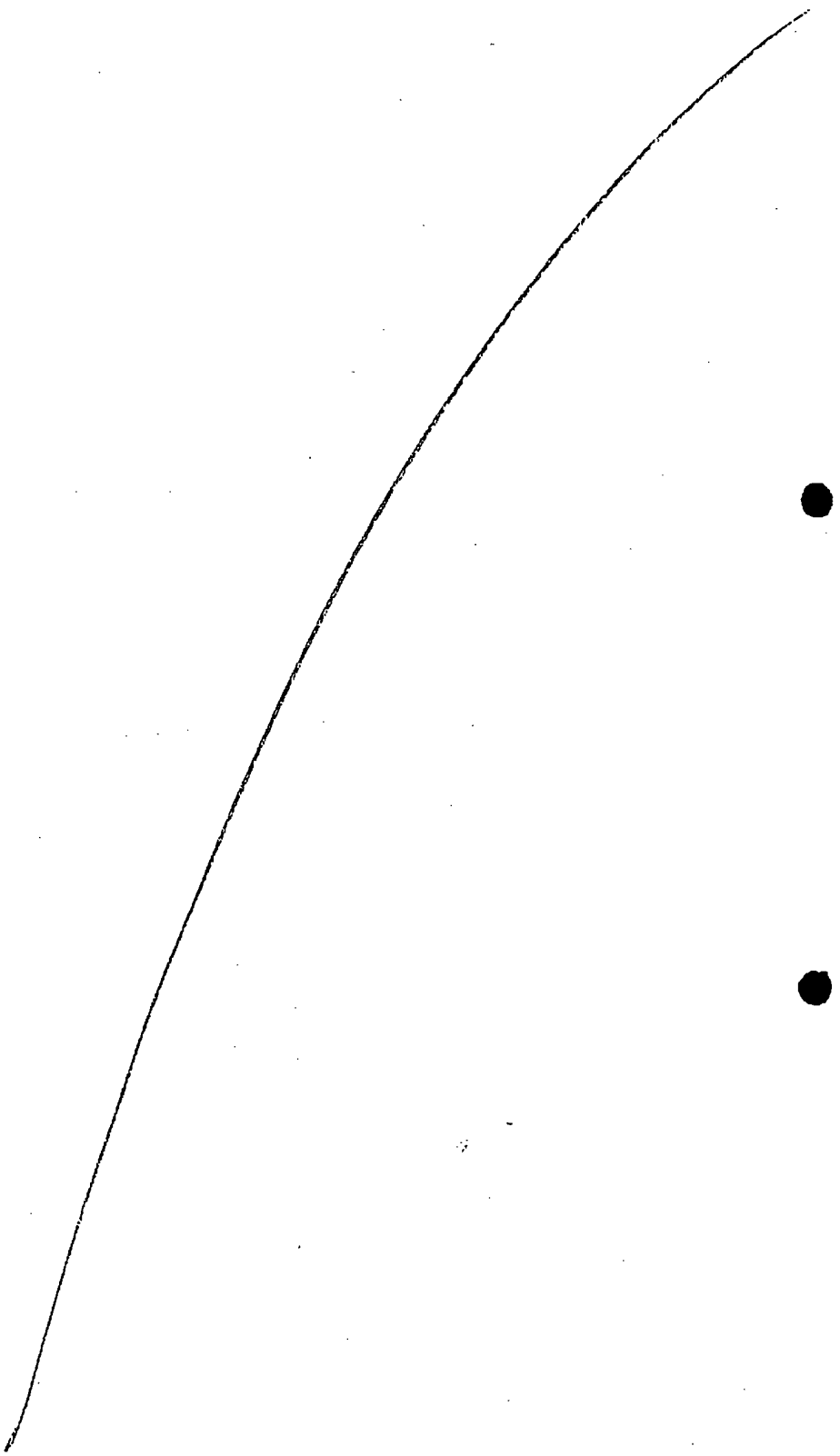
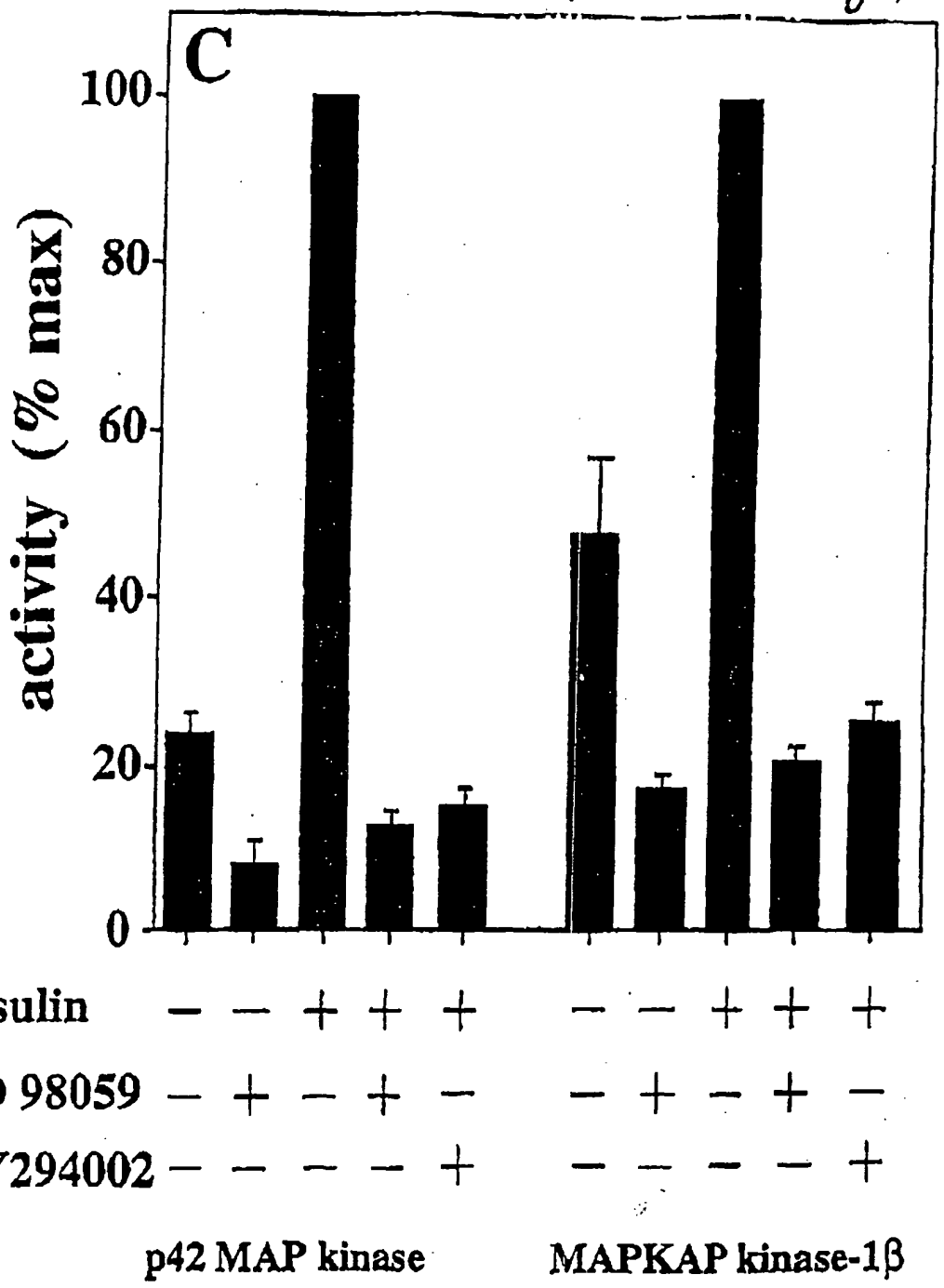


Fig 7






C
100
80
60
40
20
0
activity (% max)
Insulin – – + + + – – + + +
PD 98059 – + – + – – + – + –
LY294002 – – – – + – – – – +
p42 MAP kinase
MAPKAP kinase-1β

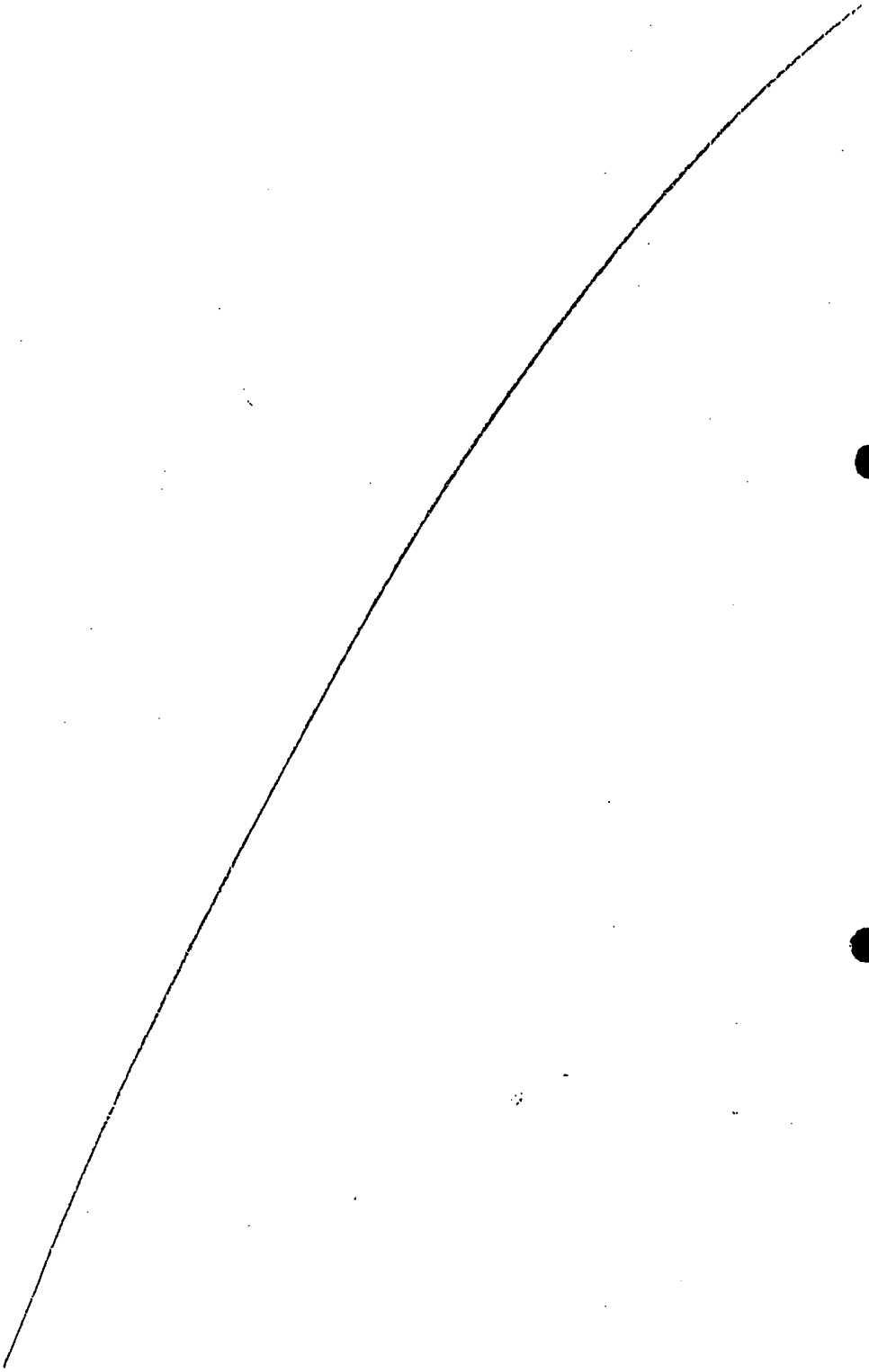
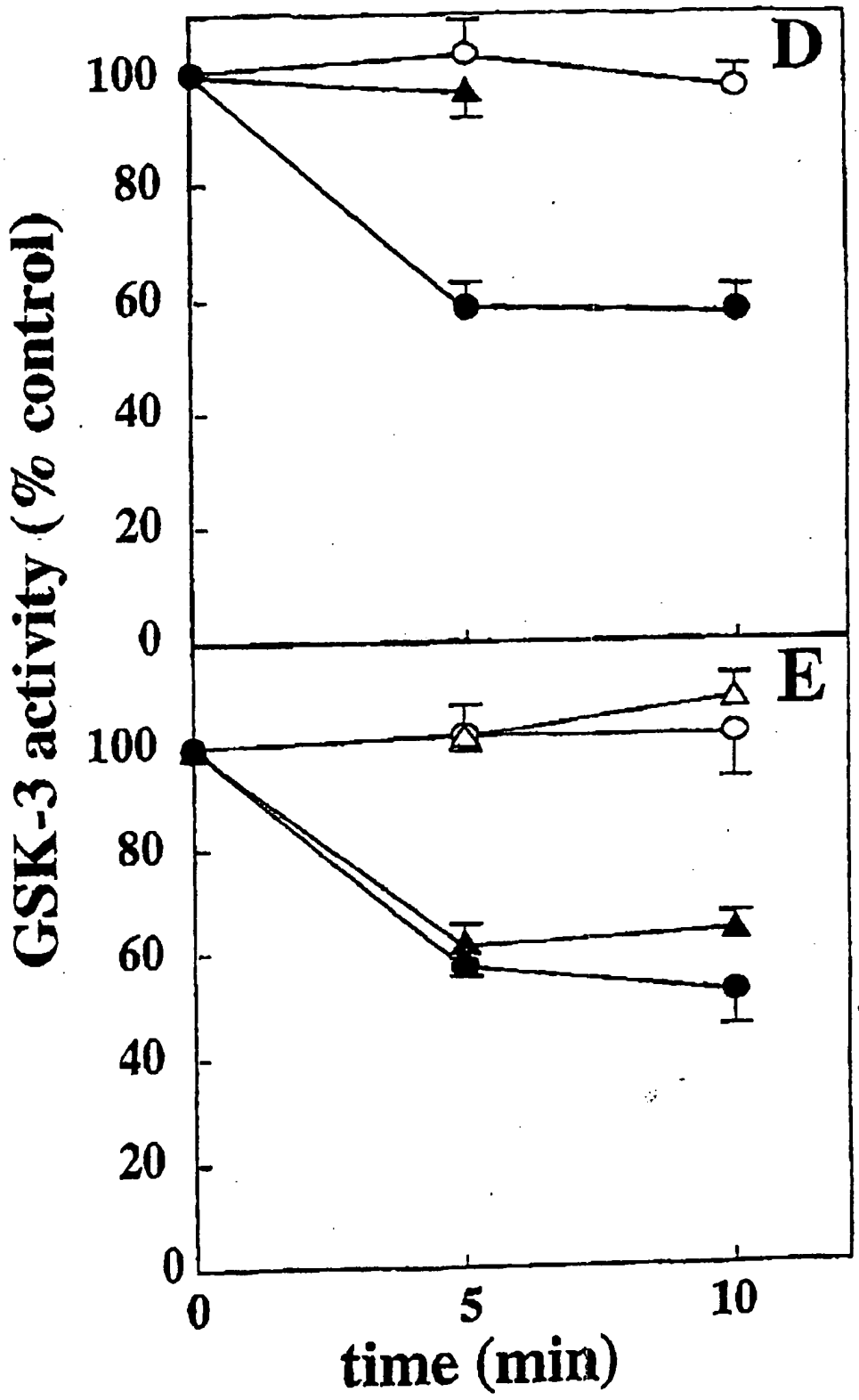


fig 9



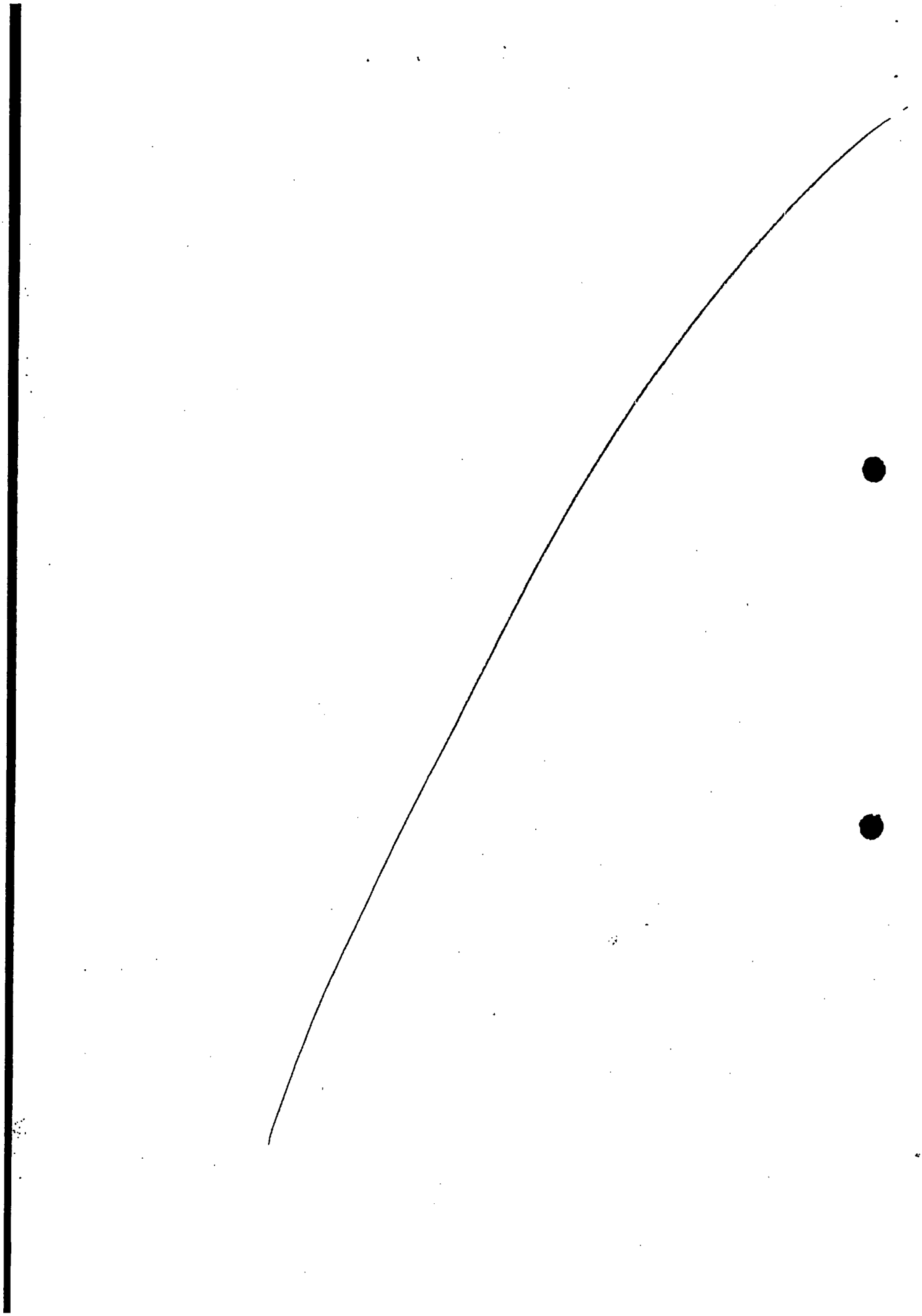
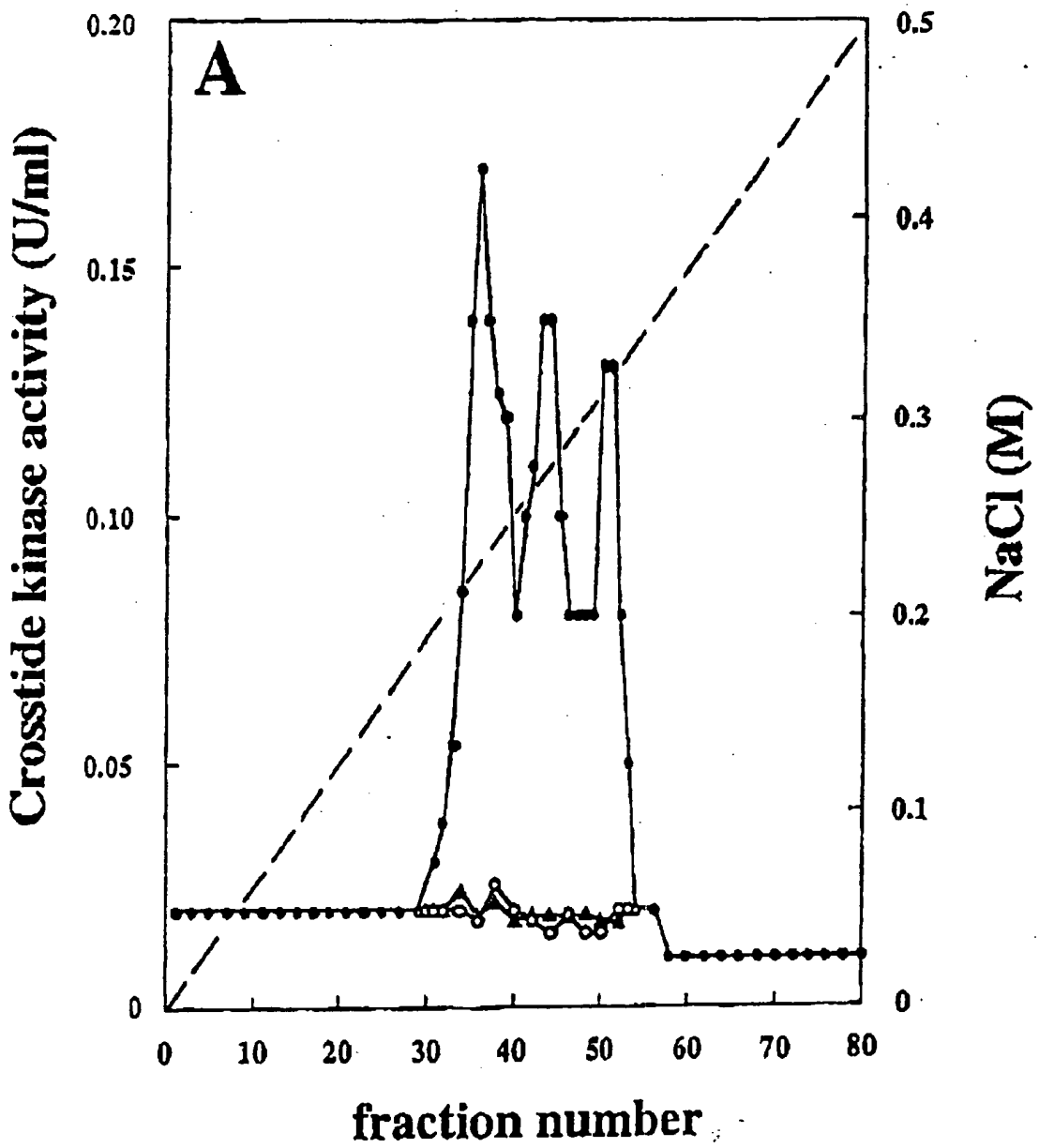


Fig 2


A
Crosstide kinase activity (U/ml)
NaCl (M)
fraction number
0.20
0.15
0.10
0.05
0
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
80

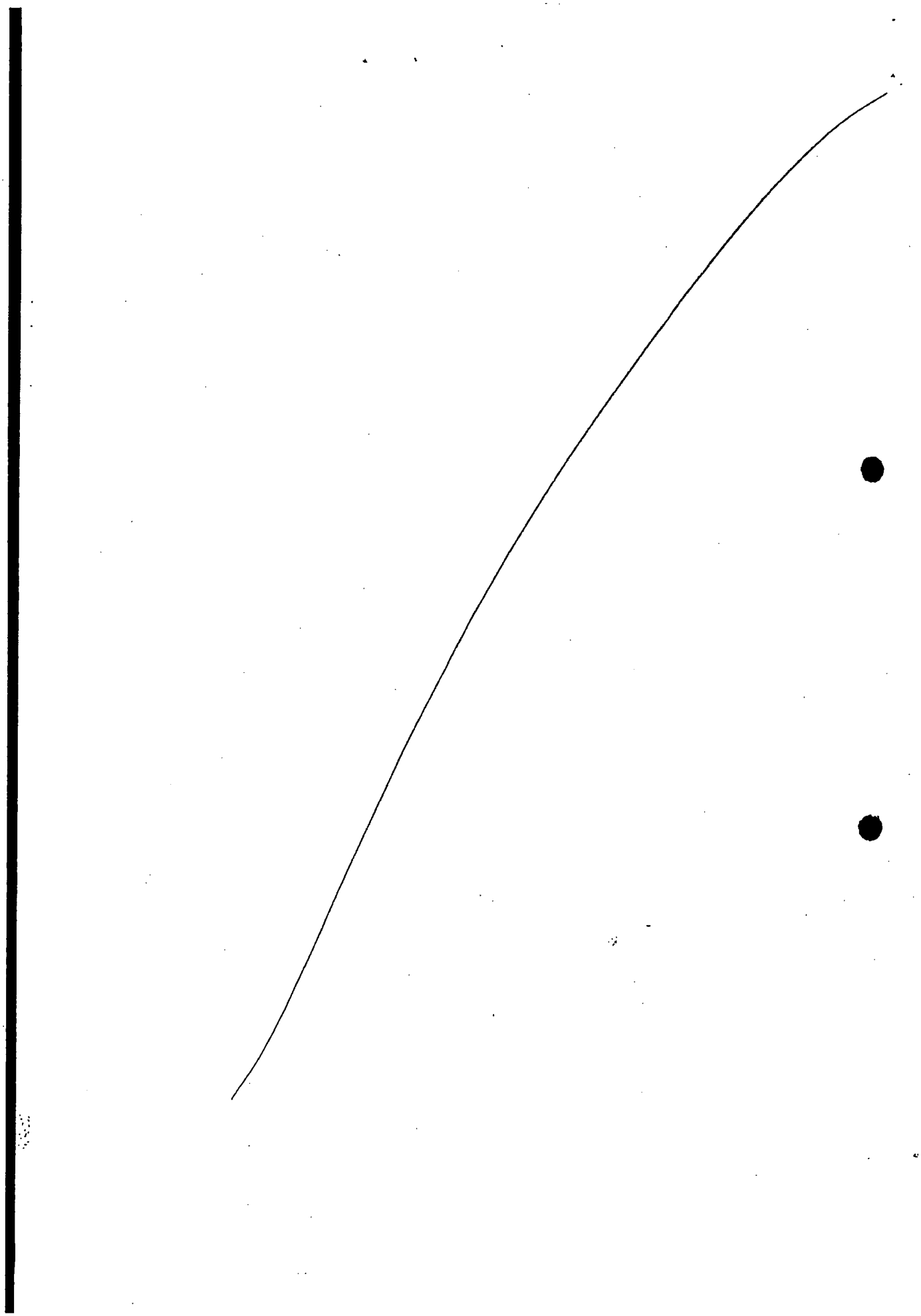
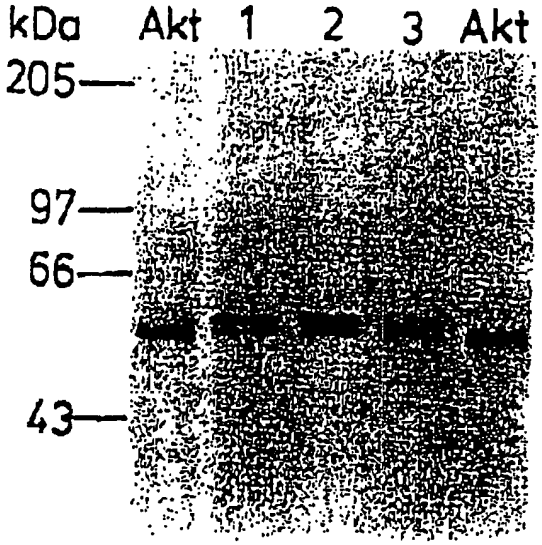
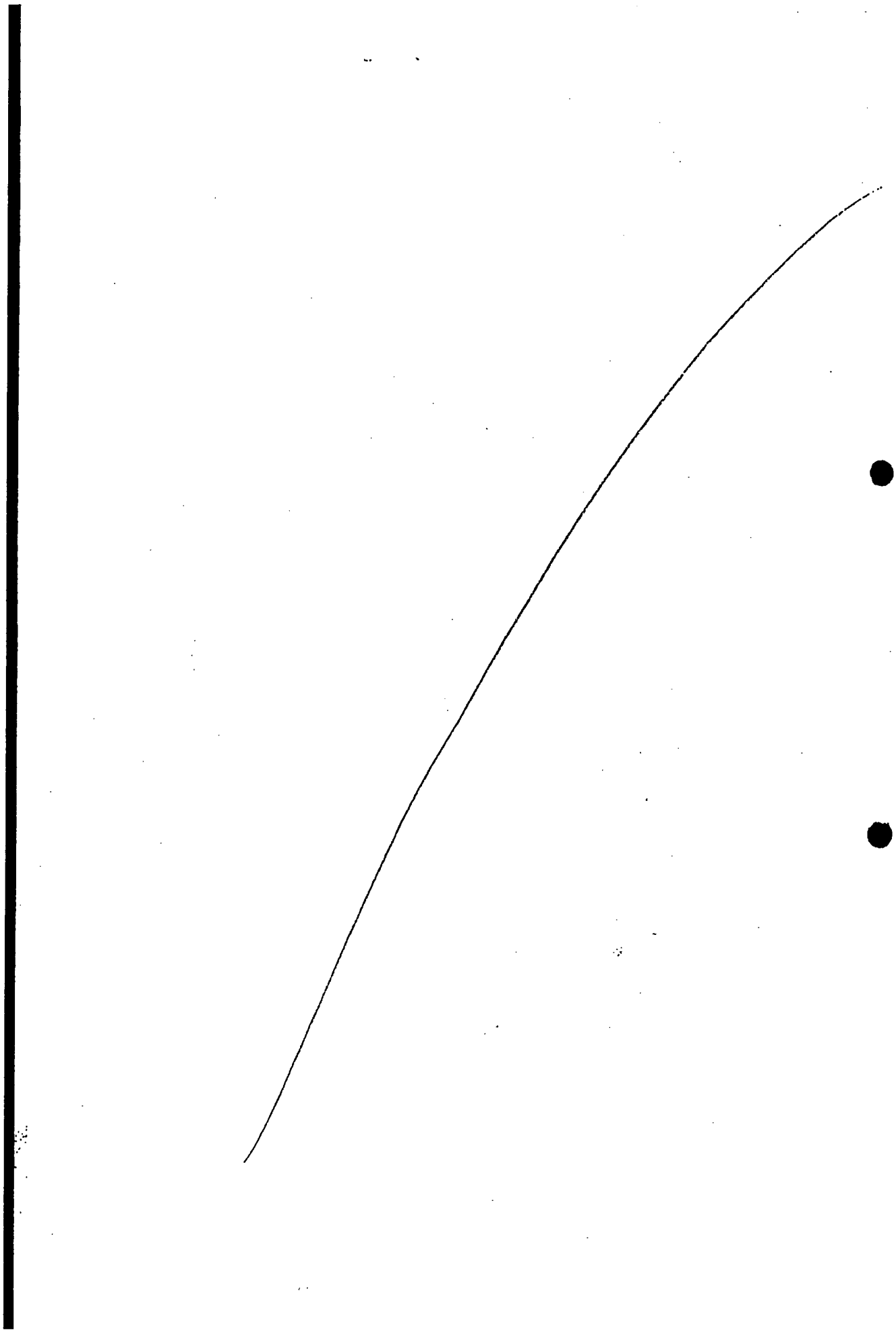
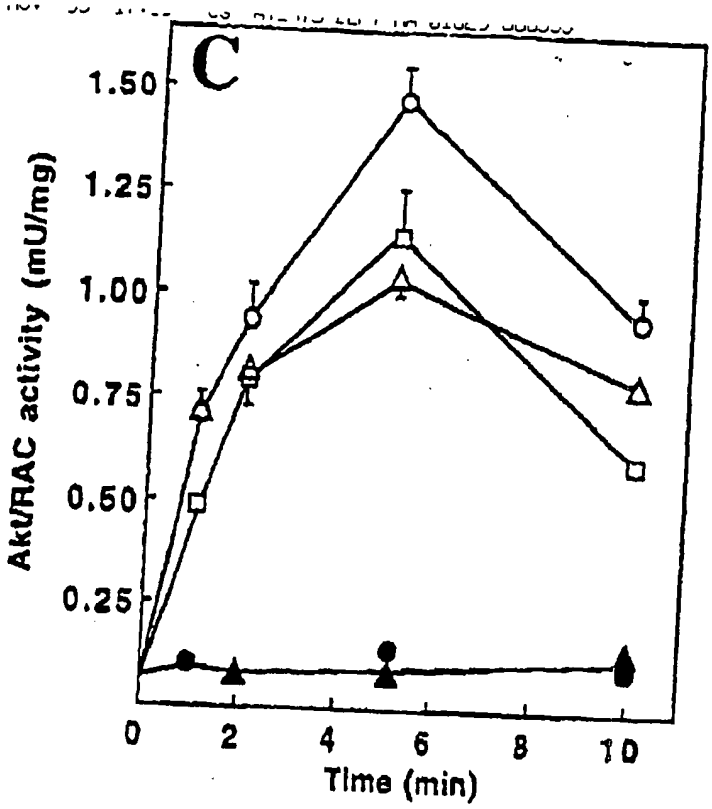
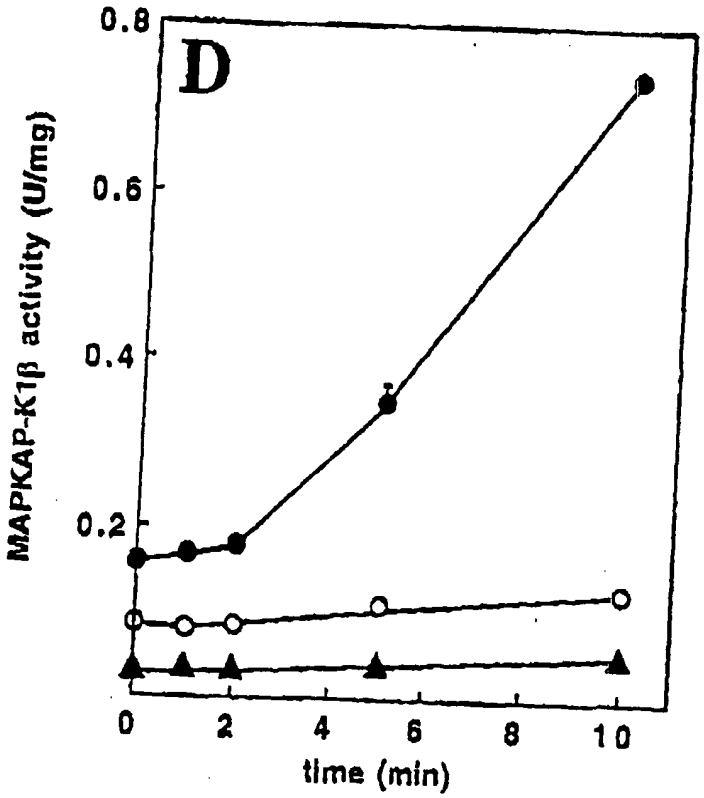


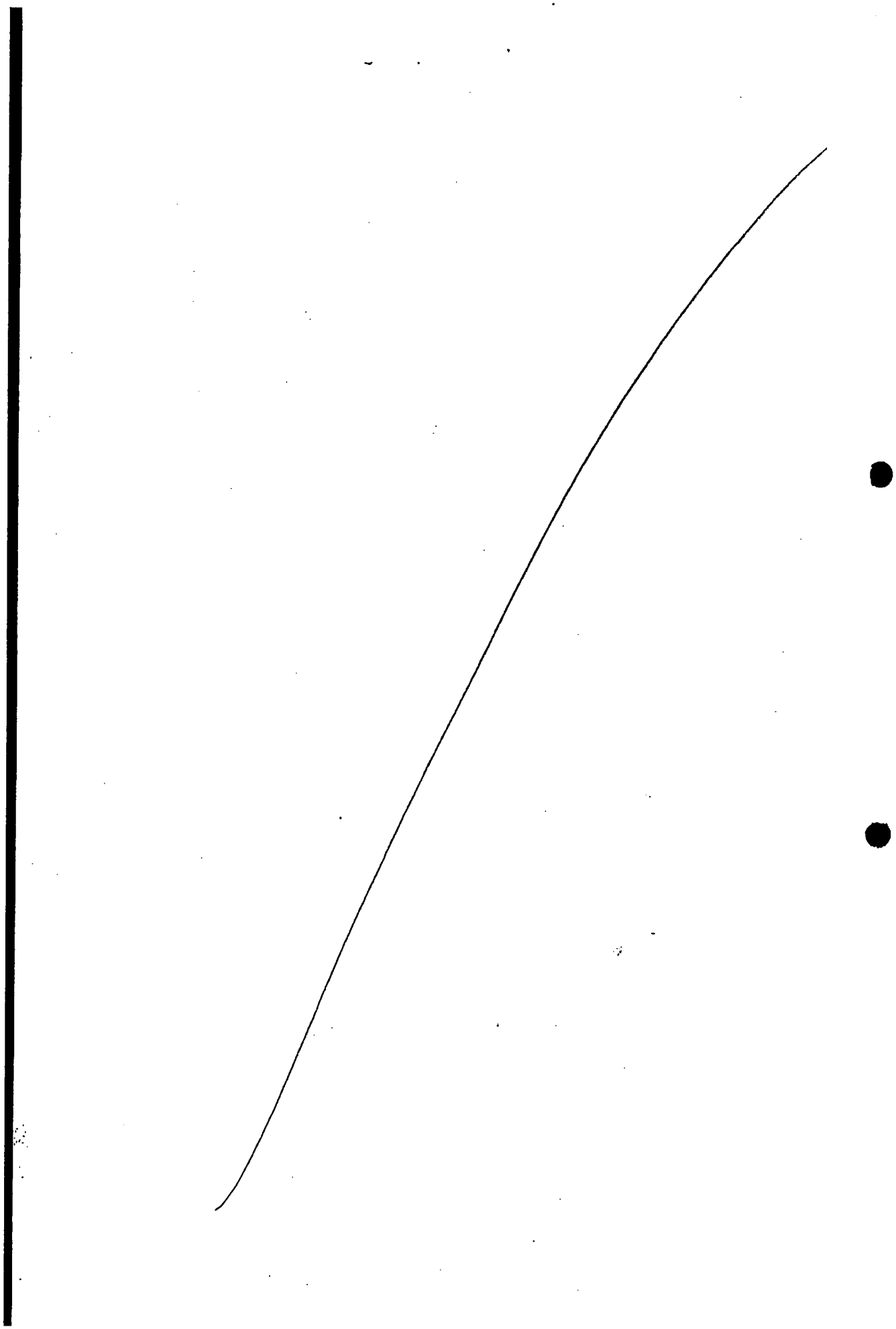
Fig 2B






C
Akt/RAC activity (mU/mg)
1.50
1.25
1.00
0.75
0.50
0.25
0
2
4
6
8
10
Time (min)


D
MAPKAP-K1β activity (U/mg)
0.8
0.6
0.4
0.2
0
2
4
6
8
10
time (min)



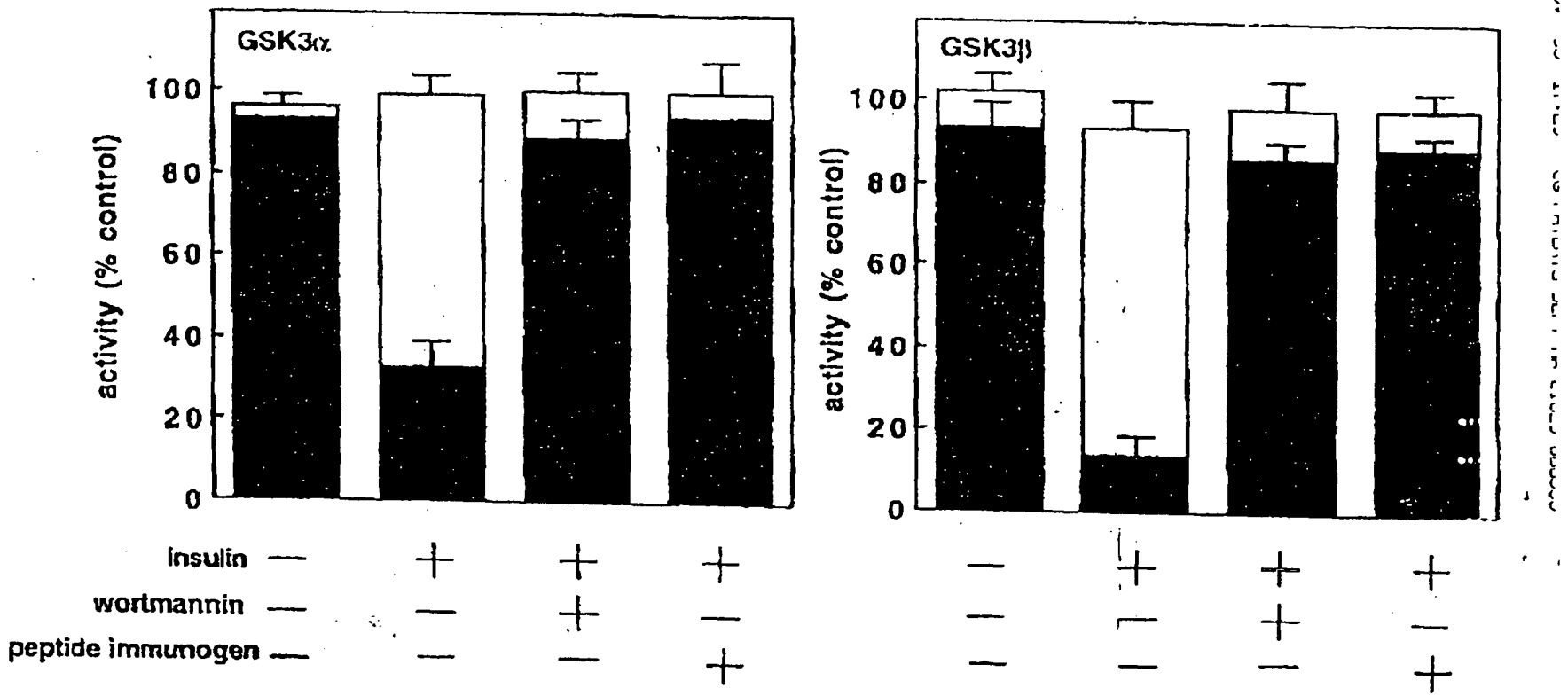
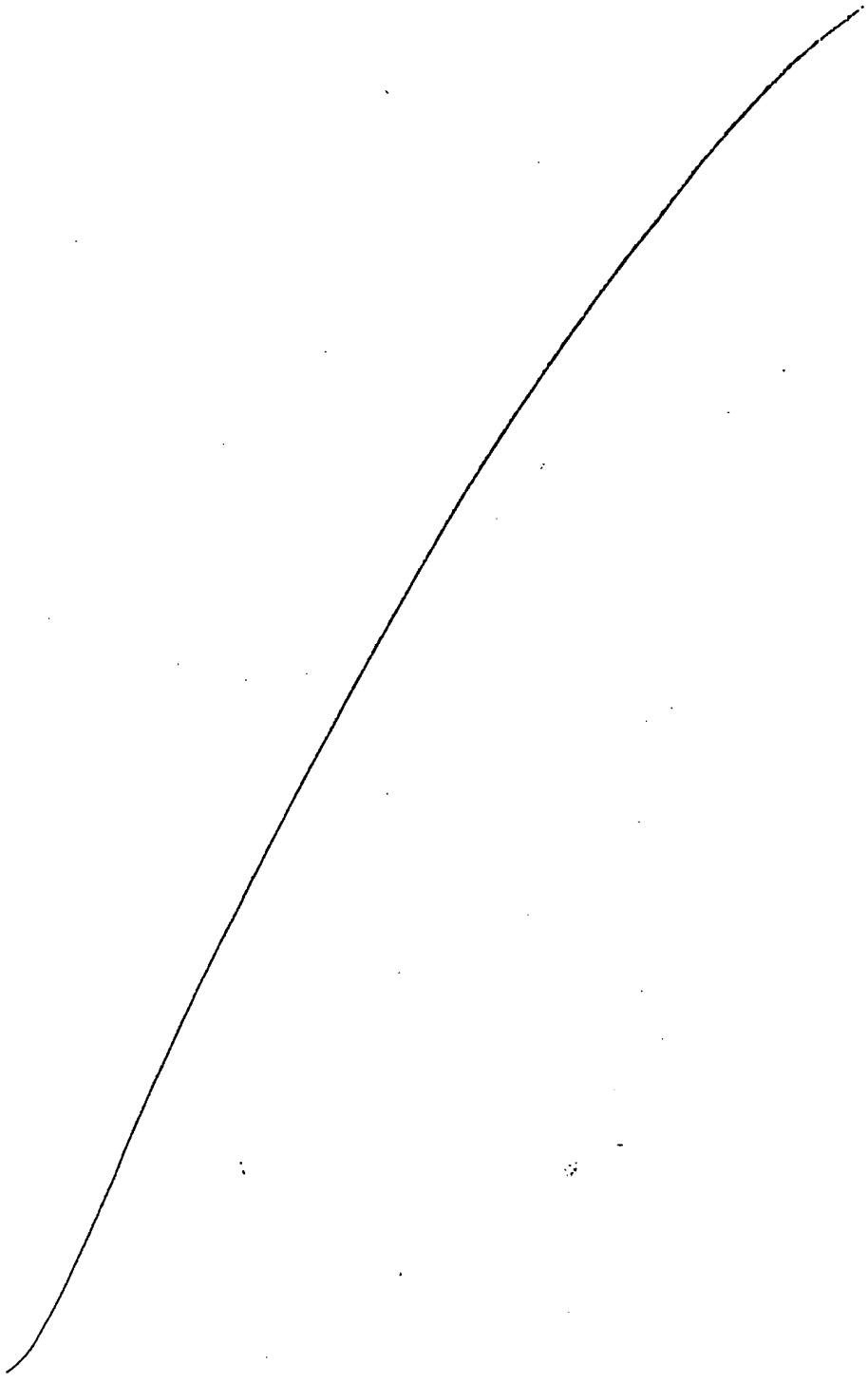

GSK3α
GSK3β
activity (% control)
100
80
60
40
20
0
insulin
wortmannin
peptide immunogen
—
+

Fig 3



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